AD_____

Award Number: W81XWH-09-1-0251

TITLE: Etiology of Sleep Disorders in ASD (Autism Spectrum Disorders): Role for Inflammatory Cytokines

PRINCIPAL INVESTIGATOR: Jessica A. Mong, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland
Baltimore, MD 21201-1531

REPORT DATE: May 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2010	Annual	1 May 2009 - 30 Apr 2010

4. TITLE AND SUBTITLE
Etiology of Sleep Disorders in ASD (Autism Spectrum Disorders): Role for Inflammatory Cytokines

5a. CONTRACT NUMBER
W81XWH-09-1-0251

5b. GRANT NUMBER
AR080087

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jessica A. Mong, Ph.D.
Email: jmong001@umaryland.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Maryland
School of Medicine
220 Arch Street
RM 02148
Baltimore, MD 21201-1531

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research Acquisition Act
Director
820 Chandler Street
Fort Detrick, MD 21702-5014

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Sleep disruptions are a common clinical feature observed in children with autism spectrum disorders (ASD). These include irregular sleep-wake patterns, delayed sleep latencies, and problems with sleep maintenance. The etiology of these sleep disturbances is unknown and remains relatively unexplored in any animal model of ASD. Prenatal valproic acid (VPA) exposure is a proposed model of ASD. Pups exposed to VPA *in utero* show similar characteristics to children with ASD, including abnormalities in brain morphology and sex-specific behavioral deficits. With this model, we examined the sleep architecture of prenatally, VPA- treated juvenile rats (PN31-34). We used a telemetry system to record electroencephalogram and electromyogram activity of each animal. Two 12-hour light phases (PN 31 and PN 34) were manually scored for each vigilance state and the data were averaged for each animal. VPA-treated animals showed a change in sleep patterning, with more consolidated bouts of wake (60.1% increase in average bout length; $t_{(11)}$=5.783, p=0.0001) and non-REM sleep (21.8% increase; $t_{(11)}$=4.066, p=0.0019) as well as fewer transitions into wake and non-REM sleep. There was a significant decrease in the number of transitions from wake to non-REM sleep (36%; $t_{(11)}$=8.657, p<0.0001), non-REM sleep to wake (30.7%; $t_{(11)}$=6.974, p<0.0001), and REM sleep to wake (52.8%; $t_{(11)}$=5.712, p=0.0001). Total sleep time and delta power, however, were similar in VPA-treated and controls animals. While these disruptions did not alter the total amount of acquired sleep in the animal's sleep phase, the alterations in patterning may disrupt sleep-dependent functions such as metabolic homeostasis, maintenance of the stress axis and synaptic plasticity. ***To our knowledge this is the first report of an ASD animal model mimicking clinical sleep disruption.*** By better understanding the consequences of disrupted sleep patterning, potential sleep therapies may be used to help alleviate some of the symptomology associated with ASD.

15. SUBJECT TERMS
Sleep architecture, Arousal, Non-REM sleep, Slow wave sleep, Valproic acid, Animal model of Autism Spectrum Disorder,

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U			UU	13	USAMRMC

Table of Contents

Introduction

Recent clinical studies report that the prevalence of sleep problems in children with Autism Spectrum (ASD) Disorders is 44% to 83% of diagnosed cases [1-4]. These sleep dysfunctions typically manifest as difficulties initiating and maintaining sleep, sleep fragmentation and insomnia. Quality sleep is imperative for the maintenance of good health. Non-ASD children and adolescents suffering from sleep disordered are not only fatigued but have impaired memory and learning, increased irritability, hyperactivity, inattention and aggressiveness leading to increased stress and anxiety and a decreased quality of daily life [5]. Children diagnosed with a ASD share similar symptoms. Thus, it is plausible to suspect that sleep disturbances in ASD may contribute to its pathology. In fact, because sustained states of hyperarousal and sleep disruption can lead to increased stress and anxiety, it is likely that disturbed sleep contributes to the development and maintenance of the aforementioned behaviors. ***A question that remains unanswered is whether the neurocircuitry underlying the sleep pathways is developmentally changed in ASD or whether disruptions in sleep are a consequence of changes in daily anxiety levels.*** Distinguishing between the two possibilities in a clinical setting would be challenging. Amazingly, rodent's neurocircuitry and neurochemistry of sleep share similarities suggesting that rats would be a good model system for basic investigations. ***Surprisingly, to date, the current animal models of ASD have not been utilized to investigate the underlying causes of sleep disturbances.*** In the current concept award, we hypothesized that alterations in cytokine function are involved in disruption of sleep states in ASD. To test this we prenatally exposed rat fetuses to valproic acid and tested (i) whether sleep patterns were disrupted, (ii) whether cytokine levels were changed and if so (iii) whether blocking the cytokines would correct the sleep and finally (iv) whether other neurochemical parameters of sleep were affected. We have found that indeed sleep patterns in the VPA model are changed compared to control animals. These results are described in detail below and to our knowledge are the first demonstration of sleep disturbances in an animal model of ASD. To date we have not found changes in cytokine levels but this investigation is continuing. Finally, we are preparing to investigate whether correcting prolonged wakefulness attenuates social behavior deficits.

Body

In the original statement of work, we outlined four tasks. These tasks, their progress and associated accomplishments are individually discussed below. As described in the original application, it is necessary to breed the animals in house because the VPA treatment is given on embryonic day 12.5. Unfortunately, due to unknown circumstances our animal colony developed breeding problems. Approximately, 4 months from the start of the project successful delivery became sporadic and eventually stopped altogether. The problem has been resolved. We requested a new room in our animal facility that was more secluded. This temporary interruption in available animals slowed our progress but we are now again generating data associated with the outlined tasks.

Task 1: Examine changes in sleep architecture in male and female rats prenatally exposed to valproic acid (VPA).

Design: In this task, pregnant dams received a single i.p. injection of 600mg/kg of sodium valproate on GD12.5, control females were injected with physiological saline. The dams were allowed to deliver and the pups were raised to postnatal day 35. At this time point, animals were surgically implanted with DSI telemeters and EEG recording electrodes were placed on the skull surface over the parietal cortex. After a recovery period (typically 5 days) the animals were reintroduced to littermate (not surgically implanted) in their homecage and the EEG acquisition was started. We collected continuous EEG data for 4 day. The EEG tracings are manually scored for sleep states. Once scored the automated software analysis parameters of sleep architecture. The results are presented in the next section.

Results:

VPA treated animals appear to have consolidated bouts of Wakefulness during their subjective night or sleep phase (i.e. Light Phase). Visual inspection of the software generated hypnograms reveal that VPA animals have longer bouts (i.e. consolidated) of wake compared to the saline treated controls (Figure 1).

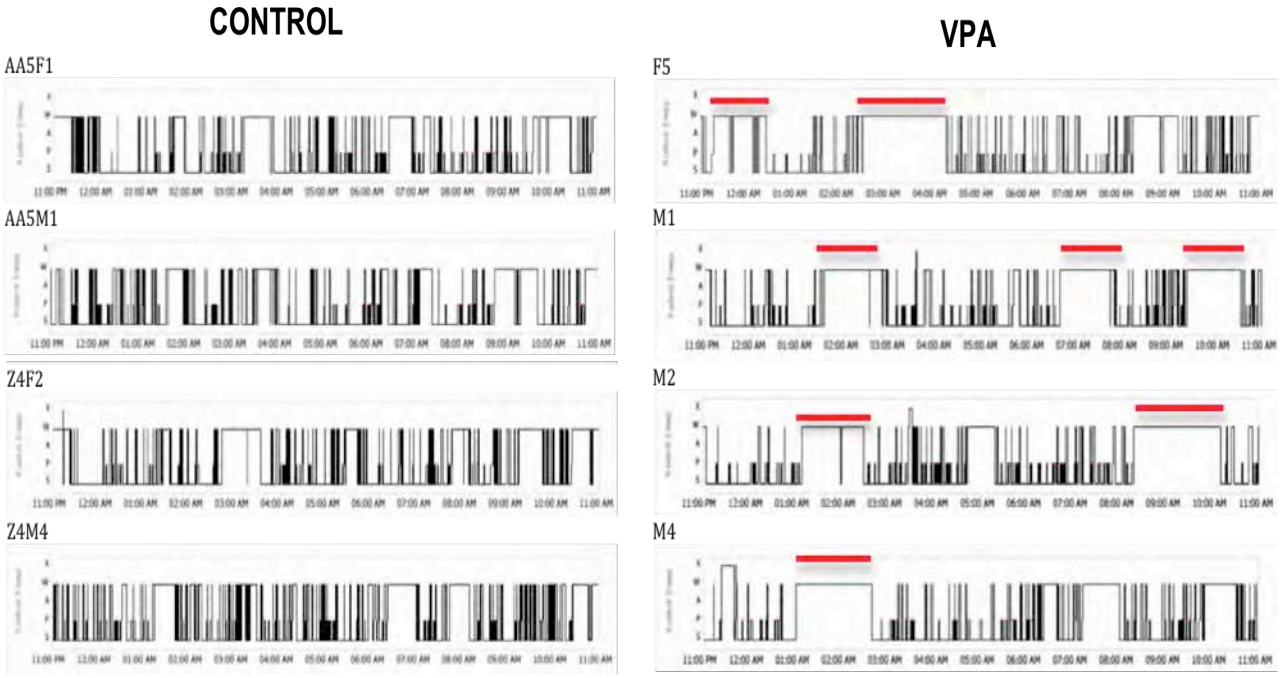

Figure 1: Hypnogram of sleep-wake behavior in the Light (sleep) phase in 4 representative animals from the Control and VPA treated groups. The x-axis represents the scored sleep states (W: Wake; P: REM as referred to as paradoxical sleep; and S: Slow wave or NREM sleep). The Red line above the Wake periods represents bouts of consolidated Wake that are statistically longer in the VPA than Control animals.

Total time spent in WAKE, Non Rapid Eye Movement (NREM) sleep, or REM sleep during the light phase was not significantly different between VPA and control treated animals. The total number of minutes spent in each state was summed across the 12 hours of light (Sleep Phase). Surprisingly, we did not detect a significant difference between VPA (n=12) or Control animals (n=12) (Figure 2; unpaired t-test)

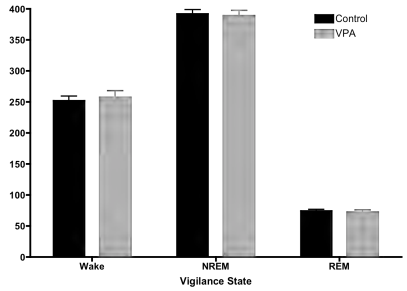

Figure 2. Total time (in minutes) spent in each vigilance state during the light phase No significant differences between controls and VPA treated animals were detected.

Total time spent in REM sleep during the Dark phase (active) was significantly different between VPA and control treated animals. The total number of minutes spent in wake, NREM and REM sleep was summed across the 12 hours of dark (active Phase). VPA treated animals spent less time in REM than control (Figure 3A; unpaired t-test $t_{(22)}$=2.636; p=0.0271). Although not significantly different, the VPA animals exhibited increased wake (Figure 3B). This observation suggest that the during the animals active phase, the VPA animals may be hyper-aroused. We are currently analyzing the activity data from these recordings to test this prediction.

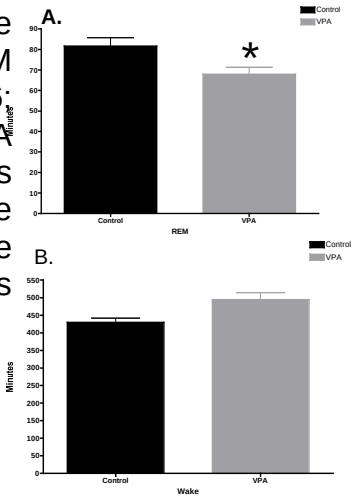

Figure 3. Total time spent in REM (A) and Wake (B) over the 12 hour dark phase. Significant difference in REM sleep were observed; VPA treated animals spent less time in REM ($t_{(9)}$=2.636; p=0.0271). These

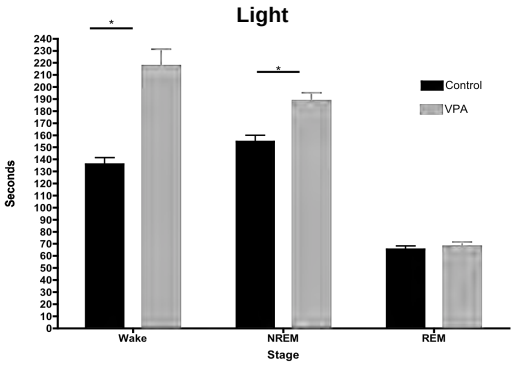

Figure 4. **Mean bout length** for each vigilance state during average 12 hour light phase (days 1 and 4 averaged). On average, wake ($t_{(22)}$=5.783, p=0.0001) and NREM ($t_{(22)}$=4.066, p=0.0019) bout lengths are significantly longer in VPA-treated animals compared to controls.

Sleep architecture was markedly different between the VPA and control animals.

Although the VPA treated animals acquired similar amounts of total wake and sleep, it was clear from the hypnograms (Figure 1) that the sleep patterns were severely disrupted. To quantify these apparent differences we analyzed the mean bout length and transitions into the various vigilance states in the light and dark phases. Mean bout length for wake and NREM sleep was significantly increased in VPA treated animals in the light phase

(Figure 4; t-test; $t_{(22)}$=5.783, p=0.0001) and $t_{(22)}$=4.066, p=0.0019, respectively). In the dark phase Wake bouts were significantly longer (t-test $t_{(22)}$=2.636; p=0.0271).

In the light phase, the mean number of transitions from Wake to N-REM, NREM to Wake and REM to Wake are significantly decreased in the VPA treated animals (Figure 5). The transitions during a 12 hour dark phase were significant differences between control and VPA treated animals.

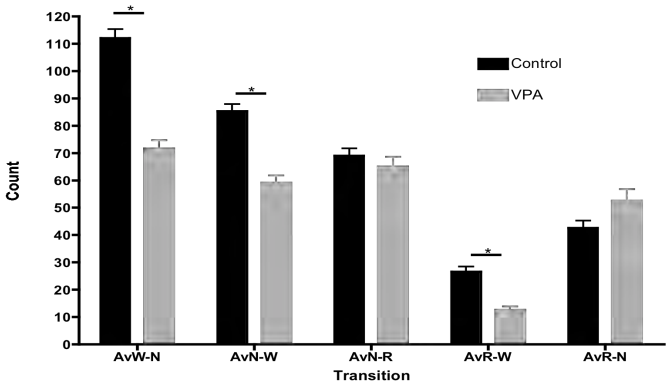

Figure 5. Mean number of transitions during average 12 hour light phase (day 1 and 4 averaged). The number of transitions from wake to NREM ($t_{(11)}$=8.657, p<0.0001), NREM to wake ($t_{(11)}$=6.974, p<0.0001), and REM to wake ($t_{(11)}$=5.712, p=0.0001) are significantly less in VPA-treated animals compared to controls

Task 2: Examine changes in cytokine expression across a developmental time period.

Design: In this task, offspring form the dams treated as described above were and are being collected over several developmental time points. Brain areas important to sleep are dissected and tissue processed for either mRNA or protein. To date, we have processed postnatal days 20, 35 and 45. The mRNA was analyzed via real-time PCR for differences in the expression of TNF-α, IL-1β and IL-10 genes and the protein extracts were run on ELISA plates for TNF-α, IL-1β and IL-10. At present, we have not detected any differences in the ages analyzed. We are currently collecting the younger cohorts to run via RT-PCR and ELISA.

Task 3: Investigate the direct role proinflammatory cytokines in sleep dysregulation in an ASD rodent model.

As we have detected any changes in Task 2, we not been able to proceed with task 3.

Task 4: Investigate the direct role of specific sleep states on ASD associated behaviors in a rodent model.

At present, we are generating animals need for this task.

Key Research Accomplishments

- In utero exposure to VPA results in sleep dysregulation in the exposed offspring.
- This VPA model will be a useful tool in further exploring the etiology of sleep disruption in ASD

Reportable Outcomes

1. Abstract to be presented in poster form at the annual Program in Neuroscience retreat for the University of Maryland, Baltimore, June 2010
2. Abstract to be presented in poster form at the Society for Behavioral Neuroendocrinology, July 2010
3. Abstract to be presented in poster form at the Society for Neuroscience, October 2010
4. Manuscript of the results reported within this report is currently being prepared for submission.

Conclusion

Taken together these data suggest that the VPA treated animals have consolidated periods of wakefulness that may be analogous to insomnia-like behavior. To our knowledge this is the first report of an accepted animals model for ASD exhibiting changes in sleep architecture that mimic that of clinical reports.

Interestingly, in one cohort of animals not included in these this analysis, we started the recordings several days prior to the reintroductions of the littermates to the homecage. When we analyzed this 'baseline' data (i.e. solitary time) we found that the differences between the VPA and control animals were not present, but when the littermates were reintroduced the increased wakefulness was observed as reported. This serendipitous finding has led us to hypothesis that the stress axis of the VPA animals is dysregulated thus leading to changes in sleep architecture. While experiments to test this our beyond the scope of the present SOW, we are currently preparing an exploratory grant to be submitted to NIH for this work.

Reference

[1] Hoffman, C., Sweeney, D., Gilliam, J., Apodaca, D., Lopez-Wagner, M. & Castillo, M. Sleep Problems and Symptomology in Children With Autism. Focus on Autism and Other Developmental Disabilities 20, 194-200, (2005).

[2] Johnson, K. P., Giannotti, F. & Cortesi, F. Sleep patterns in autism spectrum disorders. Child Adolesc Psychiatr Clin N Am 18, 917-928, (2009).

[3] Johnson, K. P. & Malow, B. A. Sleep in children with autism spectrum disorders. Curr Treat Options Neurol 10, 350-359, (2008).

[4] Johnson, K. P. & Malow, B. A. Sleep in children with autism spectrum disorders. Curr Neurol Neurosci Rep 8, 155-161, (2008).

[5] Malow, B. A. Sleep disorders, epilepsy, and autism. Ment Retard Dev Disabil Res Rev 10, 122-125, (2004).

Appendices

1. Program in Neuroscience Abstract
2. SBN Abstract
3. SfN Abstract

Program in Neuroscience Abstract

In utero exposure to valproic acid changes sleep patterns in juvenile rats: a potential model for studying sleep disturbances in autism spectrum disorders.

Danielle M. Cusmano[1,2] Michael A. Castello[2], Jessica A. Mong[2]

[1]Program in Neuroscience, [2]Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201

Sleep disruptions are a common clinical feature observed in children with autism spectrum disorders (ASD). These include irregular sleep-wake patterns, delayed sleep latencies, and problems with sleep maintenance. The etiology of these sleep disturbances is unknown and remains relatively unexplored in any animal model of ASD. Prenatal valproic acid (VPA) exposure is a proposed model of ASD. Pups exposed to VPA *in utero* show similar characteristics to children with ASD, including abnormalities in brain morphology and sex-specific behavioral deficits. With this model, we examined the sleep architecture of prenatally, VPA- treated juvenile rats (PN31-34). We used a telemetry system to record electroencephalogram and electromyogram activity of each animal. Two 12-hour light phases (PN 31 and PN 34) were manually scored for each vigilance state and the data were averaged for each animal. VPA-treated animals showed a change in sleep patterning, with more consolidated bouts of wake (60.1% increase in average bout length; $t_{(11)}$=5.783, p=0.0001) and non-REM sleep (21.8% increase; $t_{(11)}$=4.066, p=0.0019) as well as fewer transitions into wake and non-REM sleep. There was a significant decrease in the number of transitions from wake to non-REM sleep (36%; $t_{(11)}$=8.657, p<0.0001), non-REM sleep to wake (30.7%; $t_{(11)}$=6.974, p<0.0001), and REM sleep to wake (52.8%; $t_{(11)}$=5.712, p=0.0001). Total sleep time and delta power, however, were similar in VPA-treated and controls animals. While these disruptions did not alter the total amount of acquired sleep in the animal's sleep phase, the alterations in patterning may disrupt sleep-dependent synaptic downscaling possibly leading to abnormal dendritic arborization and connectivity. By better understanding the consequences of disrupted sleep patterning, potential sleep therapies may be used to help alleviate some of the symptomology associated with ASD.

Society for Behavioral Neuroendocrinology

SLEEP PATTERS ARE CHANGED IN A DEVELOPMENTAL MODEL OF AUTISM SPECTRUM DISORDER

Danielle M. Cusmano[1,2], Michael A. Castello[2], Shaun S. Viechweg[2], and Jessica A. Mong[1,2]

[1] Program in Neuroscience, [2] Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Baltimore, Maryland USA 21021

Autism spectrum disorders (ASD) are an array of developmental disorders, primarily disrupting social interactions, communication skills, and patterns of behavior. ASD is four times more prevalent in boys (4:1) than girls. Clinical observations have suggested that there is a higher prevalence of sleep disturbances in children with ASD compared to normally developing children, including difficulties initiating sleep and waking periodically throughout the night and early morning. The etiology of these sleep disturbances is unknown and remains relatively unexplored in animal models of ASD. Prenatal valproic acid (VPA) exposure is a well-accepted model of ASD. Pups exposed to VPA *in utero* show similar characteristics to children with ASD, including abnormalities in brain architecture and sex-specific behavioral deficits. With this model, we examined the sleep architecture of VPA-treated juvenile rats (PN31-38). VPA-treated rats showed a significant increase in the total time spent in wake at the expense of slow wave sleep compared to their age-matched controls. While the current data set did not allow for analysis of sex differences in VPA animals, preliminary analysis suggest a sex difference in total time spent in wake in controls. These data suggest that VPA treated animals may have disrupted sleep patterns and that a sex difference may exist in juvenile sleep. Future experiments will explore the role of hormonal and developmental factors on sleep patterning in both VPA and normally developing rats.

Society for Neuroscience

In utero exposure to valproic acid changes sleep patterns in juvenile rats: a potential model for studying sleep disturbances in autism spectrum disorders.

Danielle M. Cusmano[1,2] Michael A. Castello[2], Jessica A. Mong[2]

[1]Program in Neuroscience, [2]Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201

Sleep disruptions are a common clinical feature observed in children with autism spectrum disorders (ASD). These include irregular sleep-wake patterns, delayed sleep latencies, and problems with sleep maintenance. The etiology of these sleep disturbances is unknown and remains relatively unexplored in any animal model of ASD. Prenatal valproic acid (VPA) exposure is a proposed model of ASD. Pups exposed to VPA *in utero* show similar characteristics to children with ASD, including abnormalities in brain morphology and sex-specific behavioral deficits. With this model, we examined the sleep architecture of prenatally, VPA- treated juvenile rats (PN31-34). We used a telemetry system to record electroencephalogram and electromyogram activity of each animal. Two 12-hour light phases (PN 31 and PN 34) were manually scored for each vigilance state and the data were averaged for each animal. VPA-treated animals showed a change in sleep patterning, with more consolidated bouts of wake (60.1% increase in average bout length; $t_{(11)}$=5.783, p=0.0001) and non-REM sleep (21.8% increase; $t_{(11)}$=4.066, p=0.0019) as well as fewer transitions into wake and non-REM sleep. There was a significant decrease in the number of transitions from wake to non-REM sleep (36%; $t_{(11)}$=8.657, p<0.0001), non-REM sleep to wake (30.7%; $t_{(11)}$=6.974, p<0.0001), and REM sleep to wake (52.8%; $t_{(11)}$=5.712, p=0.0001). Total sleep time and delta power, however, were similar in VPA-treated and controls animals. While these disruptions did not alter the total amount of acquired sleep in the animal's sleep phase, the alterations in patterning may disrupt sleep-dependent synaptic downscaling possibly leading to abnormal dendritic arborization and connectivity. By better understanding the consequences of disrupted sleep patterning, potential sleep therapies may be used to help alleviate some of the symptomology associated with ASD.